REPORT DOCUMENTATION PAGE

Form Approved OMB NO. 0704-0188

1. REPORT DATE (DD-MM-YYYY)	2. REPORT TYPE	3. DATES COVERED (From - To)
23-07-2008	Final Report	15-Apr-2005 - 14-Apr-2008

4. TITLE AND SUBTITLE
Fidelity Mechanisms of DNA Polymerase alpha

5a. CONTRACT NUMBER
W911NF-05-1-0172

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER
611102

6. AUTHORS
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAMES AND ADDRESSES
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Research Office
P.O. Box 12211
Research Triangle Park, NC 27709-2211

10. SPONSOR/MONITOR'S ACRONYM(S)
ARO

11. SPONSOR/MONITOR'S REPORT NUMBER(S)
46437-LS.1

12. DISTRIBUTION AVAILIBILITY STATEMENT
Approved for public release; Federal purpose rights

13. SUPPLEMENTARY NOTES
The views, opinions and/or findings contained in this report are those of the author(s) and should not contrued as an official Department of the Army position, policy or decision, unless so designated by other documentation.

14. ABSTRACT

We have examined the mechanism by which DNA polymerase alpha discriminates between right and wrong dNTPs. With purine dNTPs, the enzyme uses a combination of positive and negative selectivity. The Watson-Crick hydrogen bonding groups at N-1 and C-6 enhance correct incorporation, whereas the electron density at N-1 and N-3 prevent misincorporation. For correct purine dNTPs, the electron density of N-3 is not essential. The exocyclic N-2 amino group of dGTP is not essential for incorporation, but does specifically prevent formation of G:A mispairs. With pyrimidine dNTPs, O-2 appears critical for incorporation. Thus, minor groove electron density appears critical for correct incorporation of pyrimidine dNTPs, but not

15. SUBJECT TERMS
DNA polymerase, fidelity, pyrimidine, purine, discrimination

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	15. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Robert Kuchta
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Standard Form 298 (Rev 8/98)
Prescribed by ANSI Std. Z39.18

Report Title

Fidelity Mechanisms of DNA Polymerase alpha

ABSTRACT

We have examined the mechanism by which DNA polymerase alpha discriminates between right and wrong dNTPs. With purine dNTPs, the enzyme uses a combination of positive and negative selectivity. The Watson-Crick hydrogen bonding groups at N-1 and C-6 enhance correct incorporation, whereas the electron density at N-1 and N-3 prevent misincorporation. For correct purine dNTPs, the electron density of N-3 is not essential. The exocyclic N-2 amino group of dGTP is not essential for incorporation, but does specifically prevent formation of G:A mispairs. With pyrimidine dNTPs, O-2 appears critical for incorporation. Thus, minor groove electron density appears critical for correct incorporation of pyrimidine dNTPs, but not purine dNTPs. Importantly, the roles of the functional groups vary substantially depending upon whether the modified base is in the template or incoming dNTP, indicating that pol alpha likely "reads" the template prior to selecting the correct incoming dNTP. Single turnover rapid quench and "trapping" approaches were developed for analyzing dNTP polymerization by pol ?. Initial experiments have indicated both that pol ? binds DNA with moderate affinity and that dNTP polymerization is only moderately fast.

List of papers submitted or published that acknowledge ARO support during this reporting period. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

Beckman, J., Kincaid, K., Hocek, M., Spratt, T., Engels, J., Cosstick, R., & Kuchta, R. D. (2007) "Human DNA Polymerase ? Uses a Combination of Positive and Negative Selectivity to Polymerize Purine dNTPs with High Fidelity" Biochemistry 46, 448-460

Number of Papers published in peer-reviewed journals: 1.00

(b) Papers published in non-peer-reviewed journals or in conference proceedings (N/A for none)

Cavanaugh, N., Trostler, M., Patro, J., Beckman, J. & Kuchta, R. D. (2008) "Mechanisms by Which DNA Polymerases Discriminate Between Right and Wrong dNTPs" Chemistry of Nucleic Acid Components, Symposium Series 14, 186-190.

Number of Papers published in non peer-reviewed journals: 1.00

(c) Presentations

4/06 FASEB Meeting, San Francisco, CA "Fidelity Mechanisms of DNA Polymerase alpha-Primase"

6/05 Gordon Conference ("Purines, Pyrimidines and Related Substances"), Newport, RI "Discrimination between Right and Wrong dNTPs by DNA Polymerases" (Presented by Kristi Kincaid)

Number of Presentations: 0.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Cavanaugh, N., Trostler, M., Patro, J., Beckman, J. & Kuchta, R. D. (2008) "Mechanisms by Which DNA Polymerases Discriminate Between Right and Wrong dNTPs" Chemistry of Nucleic Acid Components, Symposium Series 14, 186-190.

Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts): 1

Peer-Reviewed Conference Proceeding publications (other than abstracts):

Number of Peer-Reviewed Conference Proceeding publications (other than abstracts): 0

(d) Manuscripts

Number of Manuscripts: 0.00

Number of Inventions:

Graduate Students

NAME	PERCENT_SUPPORTED
Jeff Beckman	0.50
Kristi Kincaid	0.05
FTE Equivalent:	**0.55**
Total Number:	**2**

Names of Post Doctorates

NAME	PERCENT_SUPPORTED
Jen Patro	0.50
Milan Urban	0.20
FTE Equivalent:	**0.70**
Total Number:	**2**

Names of Faculty Supported

NAME	PERCENT_SUPPORTED	National Academy Member
Robert Kuchta	0.15	No
FTE Equivalent:	**0.15**	
Total Number:	**1**	

Names of Under Graduate students supported

NAME	PERCENT_SUPPORTED
Michele Loi	0.50
Alison Detweiler	0.50
Michael Trostler	0.10
FTE Equivalent:	**1.10**
Total Number:	**3**

Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period:	3.00
The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields:.....	3.00
The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:.....	2.00
Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale):	1.00
Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering:	0.00
The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense	0.00
The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields:	0.00

Names of Personnel receiving masters degrees

NAME
Total Number:

Names of personnel receiving PHDs

NAME
Kristi Kincaid
Jeff Beckman
Total Number: 2

Names of other research staff

NAME	PERCENT SUPPORTED
FTE Equivalent:	
Total Number:	

Sub Contractors (DD882)

Inventions (DD882)

Final Progress Report for W911NF-05-1-0172

The key issue we addressed was how DNA polymerase α discriminates between right and wrong dNTPs. This is a key issue in molecular recognition since all 4 bases are relatively similar chemically, and the energy difference, as measured in terms of the stability of DNA, between a correct and incorrect base-pair is much smaller than the difference in efficiencies with which a DNA polymerase incorporates a right and wrong dNTP.

1. Mechanism by which pol α discriminates between right and wrong purine dNTPs

We have identified the chemical features of purine dNTPs that human pol α uses to discriminate between right and wrong dNTPs. Removing N-3 from guanine and adenine, two high fidelity bases, significantly lowers fidelity. Analogously, adding the equivalent of N-3 to low-fidelity benzimidazole-derived bases (generating 1-deazapurines) significantly increases the ability of pol α to identify the resulting 1-deazapurines as wrong. Adding the equivalent of the purine N-1 to benzimidazole or to 1-deazapurines significantly decreases the rate at which pol α polymerizes the resulting bases opposite A, C, and G, while simultaneously enhancing polymerization opposite T. Conversely, adding the equivalent of adenine's C-6 exocyclic amine (N-6) to 1- and 3-deazapurines also enhances polymerization opposite T, but does not significantly decrease polymerization opposite A, C, and G. Importantly, if the newly inserted bases lack N-1 and N-6, pol α does not efficiently polymerize the next correct dNTP, whereas if it lacks N-3 one additional nucleotide is added and then chain termination ensues. These data indicate that pol α uses two orthogonal screens to maximize its fidelity. During dNTP polymerization, it uses a combination of negative (N-1 and N-3) and positive (N-1 and N-6) selectivity to differentiate between right and wrong dNTPs, while the shape of the base-pair is essentially irrelevant. Then, to determine whether or not to add further dNTPs onto the just added nucleotide, pol α appears to monitor the shape of the base-pair at the primer 3'-terminus.

The role of N^2 was examined by comparing a series of purine dNTPs either containing or lacking N^2 (adenine vs. 2-aminoadenine, purine vs. 2-aminopurine, 6-chloropurine vs. 2-amino-6-chloropurine). Adding the N^2 to adenine, purine, and 6-chloropurine did not significantly affect polymerization opposite G, A, or T in the template. However, adding N^2 significantly increased misincorporation opposite C (up to 100-fold enhancement). We also examined the effects of N^2 when present in the template, and the effects were analogous to N^2 in the dNTP.

Potentially, the enhanced misincorporation could have resulted from either a tautomerization/protonation of the 2-amino containing compounds, or a hydrogen bond formed between N^2 of the purine and O^2 of cytosine. To differentiate between these possibilities, we examined the effects of N^2 for polymerization opposite the base 2-hydroxypyridine (pyridin-2-one). As with a template cytidine, N^2 enhances polymerization opposite pyridin-2-one, consistent with the hydrogen bond between N^2 and O^2 driving misincorporation opposite cytidine, as opposed to tautomerization/protonation. Likewise, pol α polymerized pyridin-2-one dNTP much more rapidly opposite purines bearing N^2. To provide further evidence for the importance of this hydrogen bond, we examined polymerization of 1-deazapurine dNTP and 2-amino-1-deazapurine dNTP. Again, the presence of N^2 drove polymerization opposite template cytidine and pyridin-2-one, consistent with the hydrogen bonding mechanism.

2. Mechanism by which pol α discriminates between right and wrong pyrimidine dNTPs.

In order to test the hypothesis that pol α discriminates against incorrect pyrimidine dNTPs using similar mechanisms as it uses for discriminating against incorrect purine dNTPs, we have synthesized and tested an initial set of pyrimidine dNTP analogs (Chart 1) and measured their polymerization opposite A, C, T, and G. Pol α strongly discriminated against polymerizing dNTPs containing the bases 2-hydroxypyridine (by factors of 270, 2600, 3000, and 1800 opposite A, C, G, and T, respectively) and 2,4-dihydroxypyridine opposite all 4 natural template (by factors of 1700, >20,000, 9500, and 6200 opposite A, C, G, and T, respectively). Thus, the presence of exocyclic oxygens at C-2 and C-4 of a pyridine allow pol α to identify the incoming dNTP as wrong opposite the natural bases.

Pol α also strongly discriminated against polymerization of the dNTP containing the base 2-hydroxypyrimidine opposite A, C and T (by factors of 430, 1400, and 410, respectively). In contrast, pol α polymerized 2-hydroxypyrimidine dNTP opposite a template G only 6-fold less efficiently than dGTP. Thus, pol α recognizes 2-hydroxypyrimidine dNTP as a dCTP analogue despite the lack of a Watson-Crick hydrogen bond.

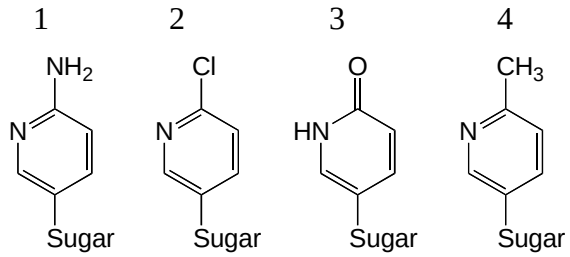

Figure 1. Bases to test the role of O^2 of a pyrimidine.

We specifically examined the role of O^2 of a pyrimidine by synthesizing 4 analogues of pyrimidine dNTPs (Figure 1). Remarkably, pol α very strongly discriminated against polymerization of all 4 dNTPs, even though in two cases the compounds could form 2 Watson-Crick hydrogen bonds. The lack of polymerization resulted from very weak binding of the dNTPs to pol α. Thus, O^2 of a pyrimidine plays a critical role during pyrimidine dNTP polymerization.

3. Functional groups play very different roles when present in the template than in the incoming dNTP.

In contrast to the very strong discrimination against the dNTPs containing the bases in Figure 1, in the case of compound 3, pol α readily incorporates dATP opposite this base when it is in the template (<10-fold discrimination). Thus, there is a huge disparity in the importance of O^2 when in the dNTP versus when in the template. We have found similar disparities in base recognition for a number of other bases (i.e., polymerization when in the template versus when in the dNTP), including 6-chloropurine, purine, and various hydrophobic bases. Together, these data indicate that pol α has the capacity to "read" the template base being replicated in order to choose the appropriate incoming dNTP. Additionally, this result helps explain why it has proven so difficult to develop novel base-pairs that DNA polymerases rapidly and efficiently replicate. Since pol α, and probably other polymerases, read the template base in order to decide what is the correct incoming dNTP, in order to know what the polymerase will consider a correct dNTP to incorporate opposite a novel template base, one must know the rules of how the polymerase reads the template base and what it will be looking for in the novel base-pair. Presently, we have no idea what the rules are – i.e., we cannot predict what the polymerase will consider correct for incorporation opposite a novel template base.

4. Pol α Does not Use "Electrostatic Complementarity" between the Bases on the Incoming dNTP and Template to Identify Correct dNTPs.

We tested the hypothesis that pol α uses "electrostatic complementarity" between the bases of the incoming dNTP and the template to help identify the incoming dNTP as right or wrong. We synthesized a series of potential novel base-pairs that would electrostatically complement each other (Figure 2). However, pol α did not rapidly polymerize any of these novel base pairs. Thus, electrostatic complementarity between the edges of the aromatic bases is not sufficient for the development of novel base-pairs.

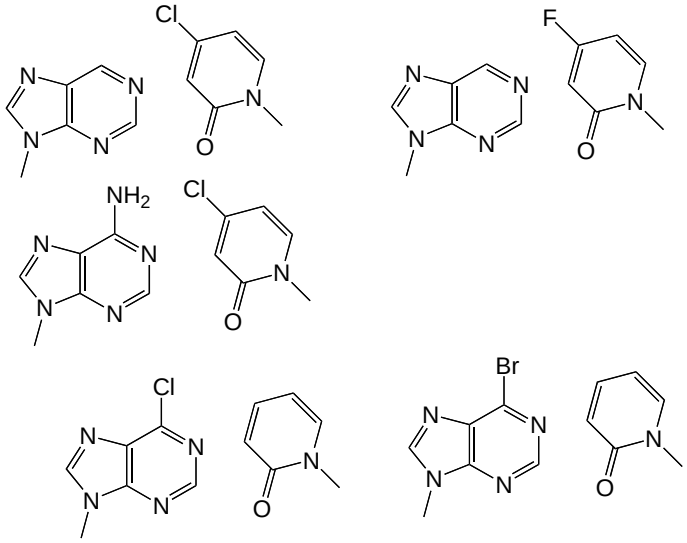

Figure 2. Potential base-pairs based on electrostatic complementarity.

5. Pre-steady state mechanism of pol α.

We first developed conditions that allowed us to perform trapping studies on pol α in order to examine a single-turnover of the enzyme. As a trap DNA, we found that poly(dT)-oligo(rA) worked very efficiently. Control experiments showed that a large excess of poly(dT)-oligo(rA) prevented free pol α from binding $[^{32}P]DNA_n$, but did not inhibit the ability of preformed E-$[^{32}P]DNA_n$ complex from adding the next correct dNTP. Using this trapping assay, we found that the E-$[^{32}P]DNA_n$ complex could add the next dNTP when diluted into trap DNA and the dNTP. Thus, like all known DNA polymerases, pol α can bind DNA prior to the dNTP. Additionally, by measuring the amount of the initial E-$[^{32}P]DNA_n$ complex formed as a function of $[^{32}P]DNA_n$ concentration, we found that the K_D of DNA is 1.5 μM for a 13/25-mer primer-template.

We have initiated rapid quench studies on pol α. A solution of pol α and $[^{32}P]$-primer/template is mixed with unlabeled dNTP and then quenched with EDTA. After concentrating the sample, the products are subjected to gel electrophoresis to separate elongated primer from unelongated primer. As with most processive polymerases, a biphasic reaction curve was obtained, indicative of a slow step after phosphodiester bond formation. From the burst phase, we can measure the rate of dNTP polymerization. As compared to replicative

polymerases from bacteria and phage, pol α is not an especially fast enzyme. The rate of dNTP incorporation is only around 40 s^{-1}.